CULTIVATION OF VACCINE VIRUS

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Many investigators have cultivated vaccine virus in bits of living tissue embedded in plasma. These small cultures were made on coverslips which were then inverted and sealed over hollow ground slides. For many reasons methods of making larger cultures are highly desirable. To this end, Carrel and Rivers (1) devised a procedure whereby cultures of vaccine virus were made in 2 to 8 cc. amounts. This was accomplished by inoculating minced chick embryo tissue with vaccine virus and then embedding it in diluted chicken plasma in Carrel flasks. This method was improved upon by Maitland and Maitland (2) who used a fluid medium consisting of minced chicken kidney suspended in chicken serum diluted with Tyrode's solution. Later Muckenfuss and Rivers (3) altered Maitland's medium by substituting rabbit testis and serum for chicken kidney and serum. The fact that certain viruses, *e.g.*, vaccine virus, Virus III, herpetic virus, will multiply in a medium of this nature has been amply verified by the work of Eagles and McClean (4), Andrewes (5–7), and Maitland and Laing (8).

The work to be described at this time has to do with the simplification of the technique for the cultivation of vaccine virus suitable for Jennerian prophylaxis in man. A medium, in which tissues and serum from halfgrown or adult animals are used, may easily permit the entry of an unknown virus into the cultures as a contaminant. The chance of such an occurrence can be lessened by the use of minced chick embryo suspended in Tyrode's solution. The possibility that such a medium would be appropriate was suggested by an experiment in which Andrewes (6) showed that Virus III survived and formed inclusions in rabbit testicular tissue suspended in Tyrode's solution.

Methods and Materials

Virus.—Levaditi neurovaccine virus that had been propagated for 6 months in rabbit testicles was used to initiate the cultures.

Tissues.—Testicular tissue was obtained aseptically from rabbits, washed in Tyrode's solution, placed in a sterile watchglass contained in a Petri dish, minced with scissors, and then distributed in proper amounts into flasks by means of a pipette. Embryo tissue was obtained from eggs, incubated 9 to 12 days, that had been opened according to the method of Carrel and Rivers (1) or that of Eagles and McClean (4).

Fluids.—When serum was used it was collected from rabbits, and diluted with 3 parts of Tyrode's solution. Tyrode's solution, pH about 7.2, prepared according to the following formula was sterilized by filtration: NaCl, 8 gm.; KCl, 0.2 gm.; $CaCl_2$, 0.2 gm.; $MgCl_2$, 0.1 gm.; $NaHPO_4$, 0.05 gm.; $NaHCO_3$, 1.0 gm.; glucose, 1.0 gm.; water *q.s.* 1000 cc.

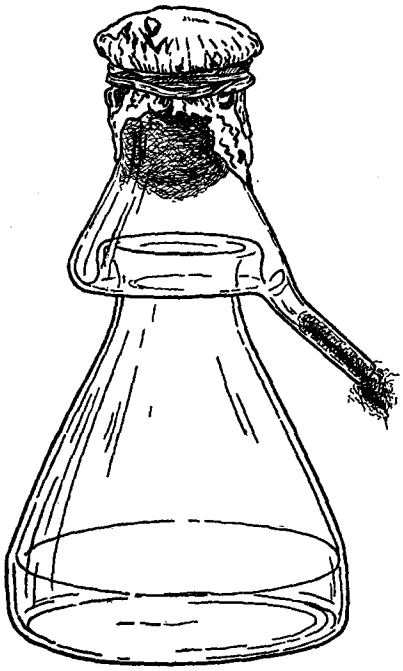
Containers.—Containers for the production of vaccine virus in large amounts must be of sufficient size and easy to handle. Furthermore, they must permit aeration and prevent contaminations and evaporation. At first, cultures were made in Carrel D flasks. Then 50 cc. Erlenmeyer flasks, stoppered with cotton over which two layers of tinfoil were placed, were used. Neither type of container was found entirely satisfactory for the work. Finally a flask was designed (collar flask) which proved to be suitable. The mouth of the flask (Text-fig. 1) is of sufficient size to permit the removal of the contents by means of a pipette. It is closed by a cotton plug over which are stretched two or three layers of tinfoil. The enlargement in the neck reduces the amount of water of condensation on or near the cotton plug and also prevents the water that does touch the cotton from running down and contaminating the culture. The small opening, plugged with cotton, on the side serves for ventilation. The bottom is perfectly flat, thus providing a large area over which a thin layer of culture may be evenly distributed. The flasks are made in two sizes. The dimensions of the large one are: height, 14 cm., diameter of mouth, 3.5 cm., diameter of base, 9 cm. The measurements of the small one are: height, 9 cm., diameter of mouth, 2.5 cm., diameter of base, 4.5 cm. In the large flasks, 2 to 4 gm. of tissue suspended in 15 cc. of Tyrode's solution were used, while in the small ones 0.5 to 1 gm. of tissue suspended in 5 cc. of Tyrode's solution was employed.

Preparation of Cultures.—Approximately 1 gm. of tissue was used to each 5 cc. of diluted serum or Tyrode's solution. Testicular or chick embryo tissue suspended in its vehicle was distributed in flasks. Prior to use, the sterility of the medium was tested on blood agar and in broth. During this interval (48 hours), the prepared flasks were stored at +5°C. The medium was then inoculated with 0.1 to 0.2 cc. of virus emulsion, and the cultures were incubated at 37°C. for 7 days. New cultures were made by the direct transfer of a few drops of the old culture into flasks of fresh medium. In this simple manner, vaccine virus can be propagated through an indefinite number of culture generations.

Titration of Virus.—Cultures for titration were ground in sterile mortars, and then appropriate dilutions were made with Locke's solution. A fresh pipette was always used for each successive dilution. 0.25 cc. of each dilution was injected into the shaved skin of rabbits. Daily observations of the animals were made for a week.

EXPERIMENTAL

In our investigation concerning the cultivation of vaccine virus, many experiments have been performed. All of them will not be



TEXT-FIG. 1. Type of flask used for the cultivation of vaccine virus

described, inasmuch as some are similar to experiments made and reported by others (8). Part of our work, however, is different from that previously described and the results are summarized in Chart 1.

The first cultures of Series A (Chart 1) were made February 17, 1930. A medium consisting of rabbit testicular tissue and a mixture of rabbit serum (1 part) and Tyrode's solution (3 parts) was inoculated with

vaccine virus and then distributed in Carrel D flasks. Virus from a culture of the third generation of Series A was inoculated into a flask (Series B) of medium comprised of chick embryo tissue suspended in

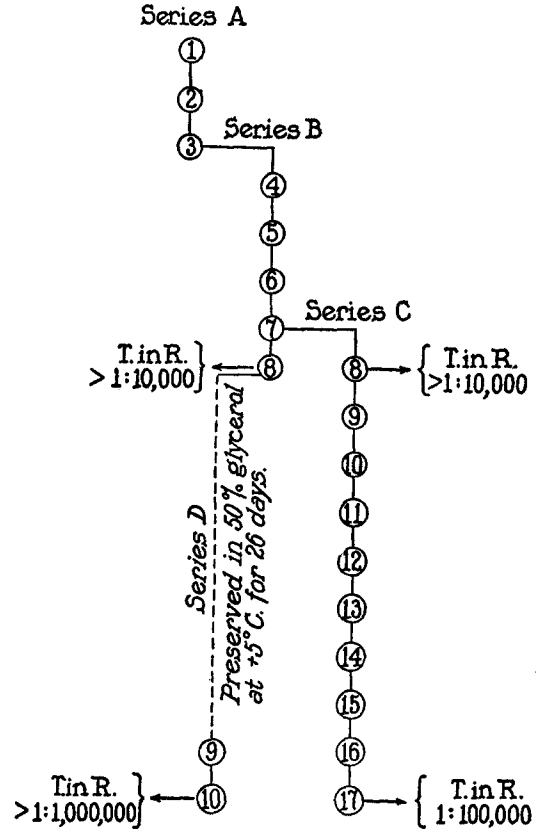


CHART 1. Summary of experiments on cultivation of vaccine virus.

The media used in the different series were as follows: Series A: Rabbit testicular tissue in a mixture of rabbit serum and Tyrode's solution. Series B: Chick embryo tissue in a mixture of rabbit serum and Tyrode's solution. Series C and D: Chick embryo tissue in Tyrode's solution.

T. in R. indicates titer in rabbit.

rabbit serum (1 part) and Tyrode's solution (3 parts). Seventh generation virus (Series B) was inoculated into a medium of chick embryo tissue suspended in Tyrode's solution (Series C). At this time collar flasks were first employed and since then they have been in general use.

The virus in Series C has been propagated for 10 generations, and is still under cultivation.

The fact that the virus multiplied is obvious and a mathematical proof seems superfluous. Nevertheless, one estimation will be given. The titer of the eighth generation virus (Series B) was >1:10,000. This virus mixed with glycerol was stored at +5°C. for 26 days. Then the preserved virus was diluted 1:100 with Tyrode's solution, and 5 drops of the dilution were inoculated into 5 cc. of fresh medium. After a 9-day period of incubation, 4 drops of this culture were added to 15 cc. of fresh medium. After incubation the titer of the last culture was 1:1,000,000 (Series D).

From the work summarized in Chart 1, it is obvious that the virus can be transferred without difficulty from one kind of medium to another. Furthermore, culture virus, mixed with glycerol, sealed, and stored at +5°C., can be preserved for a long time. In this manner virus has been preserved for 64 days without great loss in potency. Moreover, the preserved virus can be employed as an inoculum for the initiation of fresh cultures.

The virus propagated in cultures for a long time seems to have lost none of its characteristics, for, when it is spread on the scarified shaved skin of a rabbit, typical vaccinal lesions occur. Moreover, serum from a rabbit immune to Levaditi's neurovaccine virus completely neutralizes the culture virus. Finally, an immune serum prepared with the culture virus neutralizes the Levaditi vaccine virus.

DISCUSSION

A simple method for the *in vitro* cultivation of vaccine virus has been devised. The flasks employed are easy to manipulate and permit the production of large amounts of virus. Moreover, the medium is innocuous and inexpensive, consisting of minced chick embryo suspended in Tyrode's solution. In spite of the simplicity of the procedure, however, one should not forget the fact that living susceptible host cells are employed. No one has as yet been able to demonstrate that vaccine virus can multiply in the absence of such cells.

The facility with which vaccine virus can be handled and cultivated *in vitro* varies with the strain. This fact has been evidenced by previous experiences in our laboratory and also in that of Eagles and

McClean (4). In the work reported at this time a neurovaccine virus was employed. Studies have been planned to determine whether it is possible to adapt a dermovirus to the type of medium used by us.

SUMMARY

1. A strain of neurovaccine virus was cultivated in a medium consisting of minced chick embryo suspended in Tyrode's solution.

2. The virus upon cultivation apparently lost none of its essential characteristics.

3. The culture virus can be preserved and stored for long periods of time. Furthermore, the preserved virus can be used to initiate fresh cultures.

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